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Microvesicle Production After Trauma and Its Clinical Impact on Venothromboembolism

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14. ABSTRACT Polytrauma is most often caused from explosive devices and accounts for about 65 percent of injuries to our military personnel. The patients who have polytrauma are at increased risk of developing either bleeding and/or a clot in their veins which cause a life-threatening event known as venous thromboembolism (VTE). We began enrollment of patients into the study on 2 February 2011. As of 1 October 2012, we have successfully enrolled and collected blood samples on 684 patients and 64 healthy volunteers. We have thus far analyzed plasma samples of over 230 patients and 64 volunteers. In our preliminary analysis of thrombin generation and procoagulant microvesicle analysis, we have observed that thrombin generation is accelerated early after traumatic injury and there are greater numbers of procoagulant microvesicles noted after traumatic injury relative to healthy volunteers.

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Polytrauma, thrombin generation, venous thromboembolism

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Introduction: Venous thromboembolism (VTE) is a combat casualty adverse event reported to the Department of Defense. Rates of symptomatic and asymptomatic deep vein thrombosis (DVT) and pulmonary embolism (PE) in trauma patients are as high as 44 and 24%, respectively. The current guideline is that all major trauma patients receive VTE chemoprophylaxis. This practice exposes those not at risk for thrombosis to potentially serious bleeding and there are no adequate laboratory tests currently available to target anticoagulant prophylaxis to those that need it most. The **central hypothesis** of this proposal is that traumatic injury results in the release of procoagulant and pro-inflammatory factors found both in plasma and microvesicles (MVs) derived from blood cells and injured tissues. The specific Aims of this study are:

Aim 1:

- Identify cellular origins and quantitate procoagulant microvesicles (MVs) defined by cell specific markers in patients with acute traumatic injury.
- Determine the basis of differences in thrombin generation.
-

Aim 1 will be achieved through a prospective cohort study of patients with major trauma, estimate the distribution over time of procoagulant MVs concentration by cell of origin and thrombin generation.

Aim 2: Develop a predictive signature for a pre-thrombotic individual: thrombin generation concurrent with thrombogenic microvesicles.

Body: To complete aim 1, a single center prospective case-cohort study was begun on February 2, 2011. This report is the second annual report (notification of award: October 2010). The first annual report which covered Tasks and Milestones achieved during the initial funding year was submitted on 25 October 2011. Hence, this current report will cover the progress made from 26 October 2011 – 25 October 2012. In particular, the Task 3: First Year of Patient Accrual/ Milestones will be discussed in detail.

Task3: First Year of Patient Accrual/Milestones:

Patient Enrollment: As of October 1, 2012 (during the first 19 months of study), 1227 acute trauma patients were screened for study enrolment, 503 patients were excluded, and 684 acute trauma patients and 64 healthy volunteers consented and enrolled in this study. The reasons for excluding the 503 patients were: 219 met exclusion criteria, 146 declined to participate, and we were unable to obtain consents from 138 patients. We are currently awaiting response from 40 mail-in consents sent to trauma patients who were discharged prior to obtaining consents. To date, 41 patients developed VTE within three months after trauma. This is half of the number of patients we would need based on the initial power analysis performed. This is about 4 VTE patients lower than projected target number of 80 VTE patients needed to be enrolled assuming we were to finish the study as planned on December 2013. But because the proposed study required approval by DOD prior to patient enrollment, about 4 months lapsed between date of the award and patient study enrollment. Of note, 14 out of 40 patients developed VTE after hospital discharge.

Laboratory Progress:

1. *Flow Cytometer Equipment Change*: As reported in our 1/15/2012 Quarterly report, we had to switch from Canto-1 to Canto-2 flow cytometers to perform the microvesicle analysis. This decision was made after multiple attempts to fix Canto 1 equipment due to increased non-specific signals evident while running patient plasma samples. This change led to extra 3.5 months expended on re-running the patient samples on the new equipment, Canto 2. This step was taken after consulting with MV experts from within and outside Mayo Clinic. Additionally, we have identified a commercially available reference plasma, Cryocheck (Precision

Biologic, Dartmouth N.S.), which was used with every carousel of patient samples to ensure that our technique for MV analysis was consistent.

2. *Calibrated Automated Thrombinogram (CAT):* As reported in our 1/15/2012 Quarterly report, we have added a variation of the CAT assay. In addition to the performance of standard "PPP" CAT assay (5 pM Tissue Factor/ 4 uM PCPS -phospholipid), we performed, in parallel, "PRP" CAT assays using 1pM Tissue Factor only. The rationale for performing the PRP assays was to assess for endogenous phospholipid (PL) as vehicle for thrombin generation and test the hypothesis that trauma patients will have increased plasma procoagulant PL as a result of tissue injury. We recently presented our preliminary findings at the 2012 annual meeting of the American Association for the Surgery of Trauma[1]. As with the MV analysis, Cryocheck was also used as reference plasma for every micro-titer plate of patient samples processed. Between two experienced research technologists, the Coeffcient of Variation (CV) was consistently ≤7%. We also observed significant lot-to-lot variation with the "PPP" reagent (Thrombinoscope BV, The Netherlands). For example, we observed a 26% between-lot difference in thrombin peak height (nM). The data presented in the subsequent sections of this report are adjusted for lot to lot variation. (see Appendix 2)

3. Laboratory Results: CAT and MV analyses have been completed on plasma samples from 245 acute trauma patients and healthy controls; we present preliminary statistical analyses on 238 patients who had CAT analysis performed of which 174 patients had platelet derived (CD42a), procoagulant (Annexin V positive) MV analysis performed. Data are presented as median and interquartile range (IQR) unless otherwise noted; P value ≤ 0.05 was considered statistically significant.

Demographic data: The mean ± SD Age (years) and injury severity score of trauma patients were 46 ± 20 and 13 ± 10 , respectively; 74% patients were men and 5 patients died during their hospitalization for trauma.

The trauma patients had the blood sampled at baseline, 6 hours, 12 hours, 24 hours, Day 3, day of VTE (if diagnosed) and on day of discharge (DC). Based on time of patient's presentation to our Trauma Center and logistical reasons such as earlier discharge, not all patients have had their blood drawn at all these time points. Table 1 lists the procoagulant MV (# per uL plasma) and Table 2 lists thrombin generation distribution over time-points as mentioned above; data expressed as median (IQR).

Legend:
CD42a+ - platelet derived MVs
Annexin V Pos – Presence of MV phospholipid binding (procoagulant)
Lagtime – time to initial thrombin generation
Peak Height – peak thrombin activity
ttPeak – time to reach peak thrombin activity
PPP reagent - Final concentration of 5pM Tissue Factor (TF) and 4 uM PCPS (phospholipid)
PRP reagent - Final concentration of 1 pM TF

Relative to time of discharge, trauma patients have greater number of procoagulant MVs, shortened LagTime and increased Peak thrombin activity (Tables 1 and 2):

Table 1	Baseline (n=59)	6 hr (n=115)	12 hr (n=126)	24 hr (n=100)	Day 3 (n=65)	Discharge (n=72)
CD42a+/Annexin V Pos MVs (#/uL)	39 (20-80)	22 (12-42)	18 (11-35)	19 (11-31)	25 (15-43)	59 (35-82)
Any Annexin V Pos MVs (#/uL)	783 (363-1580)	299 (159-651)	237 (139-578)	229 (147-569)	343 (202-870)	332 (183-747)

Table 2	Baseline (n=116)	6 hr (n=178)	12 hr (n=203)	24 hr (n=160)	Day 3 (n=108)	Discharge (n=120)
PPP LagTime (min)	3 (2.6-3.4)	2.9 (2.5 -3.3)	3.4 (2.6-3.4)	3.3 (2.7-3.7)	3.7 (3-4.3)	4.2 (3.4-6.0)
PRP LagTime (min)	7.3 (6.3-8.5)	7.7 (6.3-9.6)	8.7 (7.3-10.4)	9.2 (7.7-11.6)	10.6 (8.4 -13.1)	12 (9.2-19.8)
PPP Peak Height (nM)	244 (213-277)	227 (202-255)	222 (194-247)	207 (177-233)	237 (203-266)	225 (152-265)
PRP Peak Height (nM)	79 (61-114)	52 (41-68)	41 (33-59)	39 (28-53)	34 (27-51)	36 (14-42)
PPP ttPeak (min)	5.5 (5-6.4)	5.3 (4.9-6.1)	5.6 (4.9-6.5)	5.7 (5-6.6)	6.0 (5.3-7)	7.4 (6-12.1)
PRP ttPeak (min)	14.7 (12.3-17.2)	16.7 (14.5-19.7)	18.6 (16.0-22.0)	18.8 (16.3-21.9)	20.3 (17.5-23.9)	22.8 (18.5-32.1)

Plasma collected from trauma patients within 2 hours of injury in the ED was compared to the blood obtained from volunteers (Tables 3 and 4). Early after trauma, there were significantly greater procoagulant MVs, decreased LagTime and increased Peak Height as compared to volunteers:

Table 3	Group	N	Mean ± SD	P- value
CD42a+/Annexin V Pos MVs (# / uL)	Volunteers	22	116 ± 173	< 0.05
	Patients	59	56 ± 60	
Any Annexin V Pos MVs	Volunteers	22	349 ± 442	<0.01
	Patients	59	1218 ± 1573	

Table 4	Group	N	Mean ± SD	P- value
PPP LagTime (min)	Volunteers	64	2.7 ± 0.4	<0.01
	Patients	116	3.0 ± 0.9	
PRP LagTime (min)	Volunteers	64	8.7 ± 1.9	<0.05
	Patients	115	7.8 ± 2.9	
PPP Peak Height (nM)	Volunteers	64	323 ± 48	<0.001
	Patients	116	241 ± 62	
PRP Peak Height (nM)	Volunteers	64	30.0 ± 13.8	<0.001
	Patients	115	90.1 ± 44.1	
PPP ttPeak (min)	Volunteers	64	5.5 ± 0.9	0.134
	Patients	116	5.8 ± 1.9	
PRP ttPeak (min)	Volunteers	64	21.1 ± 3.5	<0.001
	Patients	115	15.1 ± 4.5	

Key Research Accomplishments:

- Standardization of methods to perform the MV analysis by flow cytometry and thrombin generation by calibrated automated thrombinogram (CAT).
- Screened 1227 trauma patients and enrolled 684 patients and 64 volunteers into the proposed study.
- Plasma sample analysis of 238 patients performed each patient having an average of three samples for CAT and MV analysis.
- Through a prospective cohort study of patients with major trauma, we estimated the post-trauma procoagulant MV concentration and thrombin generation distribution over time.
- Enrolled 50% of trauma patients who developed VTE for eventual data analysis as outlined in Aim 2.

Reportable Outcomes:

- In the evaluation of the trauma patients as a group, thrombin generation is accelerated and elevated early after traumatic injury relative to their time of discharge.
- In the evaluation of the trauma patients as a group, there is greater number of procoagulant MVs after traumatic injury relative to their time of discharge.
- Reference plasma should be used in conjuction with patient samples to assess consistency in technique and methods.
- Reference plasma should be used in conjuction with patient samples to assess for any lot-to-lot variability of reagents purchased commercially.
- Poster presentation of preliminary findings at the 2012 American Association for the Surgery of Trauma (Appendix 1).

Conclusion: Over the past19 months since the study inception, we successfully enrolled and analyzed plasma samples of trauma patients. We estimated the distribution over time of procoagulant MVs by cell origin and thrombin generation (Aim 1) and found that, overall, patients are in a hypercoagulable state relative to their time of discharge. The use of chemoprophylaxis, blood transfusions and blood loss related to trauma are some of the confounders that we will need to account for while performing further analysis of our study subjects. These challenges of performing clinical studies stress the importance of standardization of laboratory techniques and the use of reference plasma during patient sample analysis.

As outlined in Aim 2, the ultimate goal of our study to develop a VTE predictive signature model for an individual who, based on the thrombin generation and MV analysis, can be stratified into low versus high risk for deep vein thrombosis and pulmonary embolism after trauma. We have thus far enrolled 40 patients out of 80 VTE cases needed based on initial power analysis. If so, bleeding complications related to chemoprophylaxis could be minimized by assessing the blood physiology of individual patients rather than utilizing population-based algorithms. Conversely, it may be determined that other patients require more aggressive chemoprophylaxis than is currently administered.

References:

1. Park MS, Owen BAL, Litwiller RD, Xue A, Harmsen WS, Jenkins DH, Heit JA. Procoagulant activity increases after traumatic injury: a case controls study. 2012 Poster presentation Kauai, Hawaii, The American Association for the Surgery of Trauma.

Appendices:

1. Reference abstract #1 for recent poster presentation, The American Association for the Surgery of Trauma 2012, Kauai, Hawaii:

Proagulant Activity Increases After Traumaic Injury: A Case-Control Study

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Introduction: Trauma is an independent risk factor for venous thromboembolism (**VTE**; odds ratio ~12). Better methods are needed to stratify VTE risk so that DVT prophylaxis can be targeted to those trauma patients who would benefit most. The calibrated automated thrombogram (**CAT**) is a global assay of plasma procoagulant factor activity as reflected by thrombin generation. In a case-control study, we tested the hypothesis that higher peak thrombin activity (**Peak**) and shorter time to peak thrombin activity (**ttPeak**; both reflecting a hypercoagulable state) are associated with acute trauma and correlate with injury severity score (**ISS**).

Methods: Venous blood collected in the Mayo Clinic emergency department from acute blunt trauma patients (cases; n=25) and from healthy volunteers (controls; n=37) was processed to citrated platelet-poor plasma for assay of plasma thrombin generation (CAT; 1pM tissue factor/$CaCl_2$), PT and aPTT, all expressed as median and interquartile range (**IQR**). The association of Peak and ttPeak with trauma and the correlation with ISS were tested using linear regression.

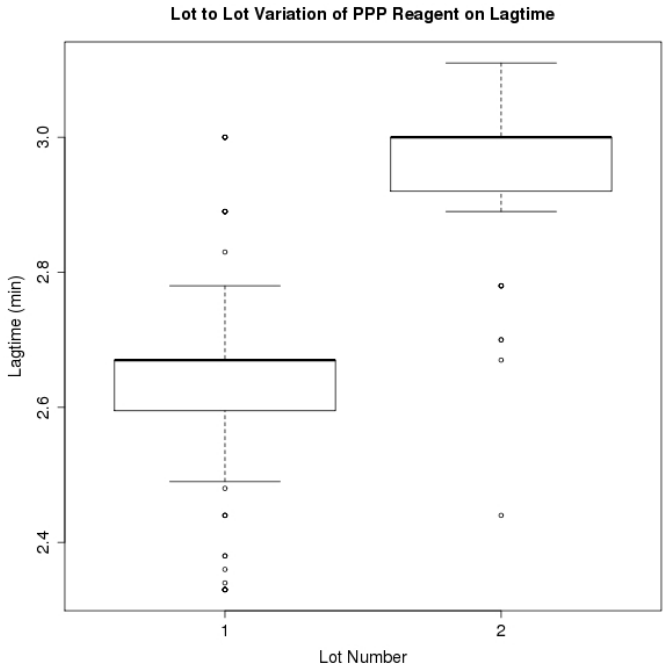
Results: The median (IQR) for patient age, ISS and time to ED after injury were 49 (22-64) years, 9 (2-17), and 57 minutes (33.5-88.0), respectively. Compared to cases, controls were significantly younger [33 (21-57) vs. 49 (19-95) years; $P<0.01$] with a higher prevalence of females (62 versus 28%; $P<0.01$). Peak and ttPeak differed significantly among cases and controls, both univariately (**Table**) and after adjusting for age and sex (PH $r^2=0.67$, $P<0.0001$; ttPeak $r^2=0.37$, $P<0.0001$), while the PT and aPTT did not. Peak and ttPeak did not correlate with ISS.

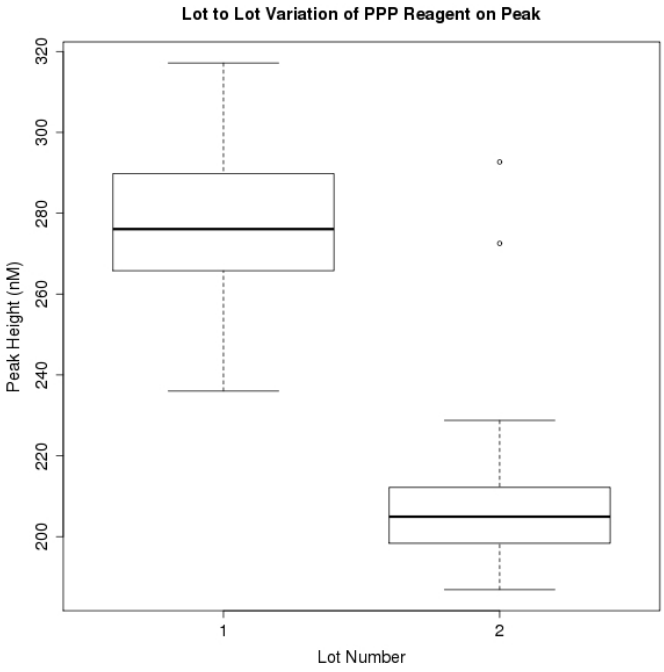
CAT Parameters	Controls (n=37)	Cases (n=25)	*P*-value
Peak Thrombin Activity (nM)	25.93 (12.80 – 59.47)	76.8 (25.6 -166.4)	<0.0001
ttPeak Thrombin Activity (minutes)	20.56 (16.33-28.56)	14.89 (97.6-23.9)	<0.0001

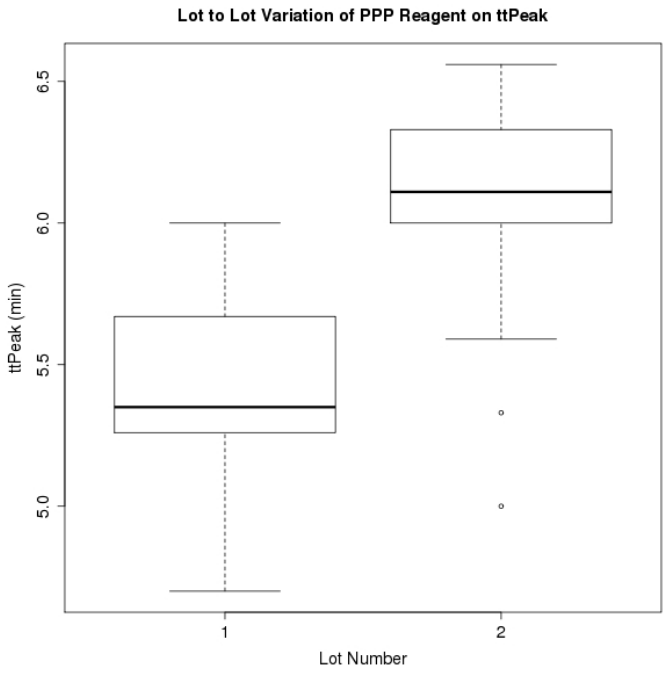
Conclusions: Blunt trauma is associated with a hypercoagulable state, as reflected by a higher peak and shorter time to peak thrombin activity. In an ongoing prospective case-cohort study, we will test the hypothesis that peak and time to peak thrombin activity are independent predictors of VTE after acute trauma. If proven correct, peak and time to peak thrombin activity may be useful for stratifying trauma patients into high- and low-VTE risk, and for targeting DVT prophylaxis.

2. Lot to lot variation of PPP reagents sold by Thrombinoscope BV accounted for during calculation of CAT parameters: Lag Time (min), Peak Height (nM) and ttPeak (min). The Graphs depicts the adjusted CAT results of Cryocheck reference plasma

a) Lot-to-lot variation between lot #1 (original) and lot#2 (new)







b) After adjustment: